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"Effect of Dietary Factors upon Intestinal Alkaline Phosphatase."

NESTER DICKIE

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Abstract

The effect of various dietary factors upon intestinal alkaline phosphatase of rats was studied. Short-chain saturated fatty acids and a long-chain unsaturated fatty acid elevated significantly the intestinal enzyme of fasted rats. Supplements of choline in the diet produced a diminution in alkaline phosphatase response. Various hexoses fed to fasted rats were able to elevate the enzyme significantly to various degrees; the effect with glucose was the greatest. In contrast, pentoses and sucrose were inactive in this regard. From a variety of proteins tested, the phosphoproteins, casein and vitellin, produced significant responses; all others were without effect.

The intestinal cells of the male rat were fractionated completely into four fractions by the method of differential centrifugation. A large per centage of the total alkaline phosphatase activity of the intestinal cell was found to be associated with the "microsome fraction". The specific activity of this fraction was several times that of the unfractionated homogenate.

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THE EFFECT OF DIETARY FACTORS UPON INTESTINAL ALKALINE PHOSPHATASE

A DISSERTATION
SUBMITTED TO THE SCHOOL OF GRADUATE STUDIES
IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE
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DEPARTMENT OF BIOCHEMISTRY

by
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I. INTRODUCTION

Alkaline phosphatase is generally defined as a phosphomonoesterase which catalyzes the hydrolysis of a variety of orthophosphoric acid esters at an optimum pH of 9.2 to 9.6 (1). Zinc and magnesium ions (2), and certain amino acids (3) activate the enzyme. Gomori (4) has indicated on the basis of magnesium activation that intestinal alkaline phosphatase consists of three components. Two of the components were found by him to become rapidly inactivated by substrate in the absence of magnesium. Alanine and magnesium activated each component to a different extent. Cloetens (5), on the basis of inhibition studies, considered two alkaline phosphatases to be present in animal organs. Akamatsu and Aso (3) reported that amino acids activate the apoenzyme of alkaline phosphomonoesterase through the formation of an amino acid-magnesium-apoenzyme complex. In further work along this line, these authors indicated that certain di-peptides and tri-peptides were able to behave in this way as well (6). Other workers (7) found that electrodialysis of kidney phosphatase inactivated the enzyme, and suggested that a dialyzable co-factor is necessary for enzyme action.

Alkaline phosphatase is found in most mammalian tissues but appears to be highly concentrated in tissues that are of a secretory nature, viz., intestinal epithelial cells of most mammals, birds, amphibians and fishes; secretory borders of the cells of the proximal tubules of the kidney; placenta, choriod plexus, and the thyroid and adrenal glands of mammals (8). The functional importance of alkaline phosphatase has been investigated. Robison (9) has ascribed a role to the enzyme in the calcification of bone. Absorption of sugars from the convoluted tubules of the kidney is considered by many workers to involve alkaline phosphatase (10 - 13). The high concentration of the enzyme in the intestinal mucosa of mammals has led to the belief that it functions in the absorption of glucose and fatty acids (14 -17).

The close correlation between sites of secretion and alkaline phosphatase has led Danielli (8) to propose a theory of phosphatase function. The theory is based upon the suggestion of Goldacre (18) that active transport processes involve contractile protein systems similar to the actomyosin-ATP system in muscle fibres. Danielli theorizes that "alkaline phosphatase is acting as the enzymic center through which chemical energy is converted into mechanical work through the mechanism of a contractile protein". He suggests that a close

relationship between alkaline phosphatase and contractile systems can be considered if one accepts the idea that active transfers are based upon contractile proteins. Since alkaline phosphatase is a non-specific enzyme, Danielli believes it may function in general transfers across the mucosa. For specific transfers, a specific enzyme would be required.

Work in this department has previously involved the study of the effect of diet upon serum alkaline phosphatase. Recently, the study has been extended to include the intestinal enzyme. Cantor, Tuba and Wight were able to demonstrate that the fat content of the diet determined the level of the serum enzyme (19). A positive correlation was found to exist between food consumption and serum alkaline phosphatase (20). Extending their work to the intestinal enzyme, these authors were able to show by inhibition studies that in animals made diabetic or fed high fat diets the serum alkaline phosphatase was derived from the intestine (21). They concluded that a major portion of the serum enzyme is derived in this way. Tuba and Robinson (22) have presented evidence that the rat intestinal alkaline phosphatase consists of an adaptive and a non-adaptive fraction. The adaptive fraction was shown to respond to the feeding of fat and was considered by

these authors to be responsible for the elevated serum levels of alkaline phosphatase. The study of the effect of fatty acids in the diet upon the intestinal enzyme was begun (23) and is continued in this thesis.

II. METHODS

1. Reagents

(i) Substrate-buffer mixture:

0.4240 gm. sodium diethyl barbiturate
0.5000 gm. sodium beta glycerophosphate
0.2464 gm. magnesium sulfate heptahydrate

dissolved in 100 ml. double distilled water.

(ii) 10% trichloracetic acid.

(iii) 0.1 N sodium hydroxide.

(iv) Molybdic acid:

Stock solution: 7.5% sodium molybdate
10 N sulfuric acid

Working solution: one part of 7.5% sodium molybdate added to one part 10 N sulfuric acid with constant shaking. Solution to be used shortly after preparation.

(v) Stannous chloride:

Stock solution: 6.0 gm. stannous chloride and 10 ml. concentrated hydrochloric acid. Covered with a layer of toluene and stored at 5° C.

Working solution: 0.2 ml. of stock solution diluted to 100 ml. with double distilled water. Used within four hours.

(vi) Standard phosphorus solution:

Stock solution: 0.4394 gm. potassium acid phosphate made up to 1 liter with distilled water.

Working solution: 2 ml. of stock solution is diluted to 100 ml. with distilled water. One ml. contains two micrograms of phosphorus.

2. Diets

A. Carbohydrates:

(i) Cellulose.

General Biochemicals,Inc..

(ii) D-Glucose.

C.P. Anhydrous. Merck and Co.

(iii) D-Fructose.

Crystal. Practical. Pfanstiehl Chemical Co.

(iv) Sucrose.

Crystal. Analar. British Drug Houses Ltd.

(v) D-Galactose.

Practical. Pfanstiehl Chemical Co.

(vi) D-Arabinose.

C.P. Pfanstiehl Chemical Co.

(vii) D-Xylose.

C.P. Pfanstiehl Chemical Co.

(viii) D-Mannose.

C.P. Pfanstiehl Chemical Co.

B. Fatty Acids:

(i) Butyric acid.

Technical. J.T. Baker Chemical Co.

(ii) Lauric acid.

Crystals. Eimer and Amend Chemicals.

(iii) Palmitic acid.

C.P. Crystals. Fisher Scientific Co.

(iv) Stearic acid.

U.S.P. 1X. Powder. Merck and Co.

(v) Oleic acid.

Redistilled. British Drug Houses Ltd.

C. Proteins:

(i) Casein.

"Vitamin-free". Nutritional Biochemical Corp.

(ii) Wheat gluten.

Nutritional Biochemicals Corp.

(iii) Zein.

Nutritional Biochemicals Corp.

(iv) Gelatin.

Nutritional Biochemicals Corp.

(v) Egg Albumin.

Nutritional Biochemicals Corp.

(vi) Lactalbumin.

Nutritional Biochemicals Corp.

(vii) Vitellin.

Prepared and purified from egg yolk by Dr. J. Tuba, Dept. of Biochemistry.

(viii) Bran.

8% fat, determined by extraction. Animal Science, University of Alberta.

3. The Experimental Animal

Adult male albino rats were used in all experiments. They were maintained on a diet of Purina Fox checkers prior to selection for experiment. After selection, the animals were weighed, housed individually in metal cages, were given tap water to drink ad libitum, and fasted, usually four to five days unless otherwise stated. The fasted animals were then offered various diets at 4:30 P.M. An estimate of the water consumed during the experimental period was noted. Individual consumptions and body weights were recorded before the animals were sacrificed. The animals were killed by decapitation. Whenever serum enzyme levels were to be determined, blood flowing from the trunks of decapitated rats was collected. Serum was separated from the formed elements of the blood by centrifugation and was stored at 5° C. Serum phosphatase values were determined within 48 hours after collection. For intestinal alkaline phosphatase determinations, the first ten cm. of intestine from the pylorus was excised

and quickly placed in a small beaker cooled by an ice-bath. The tissue was frozen overnight. In the morning, the piece of gut was washed out by a stream of cold, distilled water from a water bottle, dried by light blotting on filter paper, and weighed to the nearest hundredth of a gram. The portion of intestine was then homogenized in 100 ml. of cold distilled water by means of a Potter-Elvehjem glass homogenizer (24) cooled by an ice-bath. The homogenate was left overnight in the refrigerator at 5° C. In the morning the homogenate was made up to 250 ml. with distilled water. Alkaline phosphatase was determined on a 1:10 dilution of the supernatant obtained by centrifugation.

4. Phosphatase Determination

Alkaline phosphatases of the serum and intestine were determined by a macromethod based upon the micromethod of Shinowara, Jones and Reinhart (25) as modified by Gould and Schwachman (26). Phosphate ion liberated from sodium beta glycerophosphate is measured by the method of Kuttner and Lichtenstein (27). The unit of phosphatase activity is defined by Shinowara et al as "equivalent to one milligram of phosphorus liberated as phosphate ion during one hour of incubation at 37° C. with a substrate containing sodium beta glycerophosphate, hydrolysis not exceeding 10% of the

substrate, and the pH optimum of the reaction mixture for the alkaline enzyme at 9.3±0.15".

Inorganic phosphorus content of the serum or of the intestinal homogenate was determined on 0.5 ml. of a 1:10 dilution. Alkaline phosphatase was determined with 0.5 ml. of a 1:100 dilution of serum, and 0.5 ml. of a 1:10 dilution of the supernatant from centrifuged intestinal homogenates. The diluted serum or supernatant was pipetted into 13x100 test tubes which were then placed in a water bath and warmed to 37° C. One ml. of distilled water was added to the tubes containing serum or supernatant to be tested for inorganic phosphorus. One ml. of the substrate-buffer was added to the tubes containing serum or supernatant to be tested for phosphatase. Hydrolysis was allowed to proceed for exactly one hour. The reaction was stopped by the addition of one ml. of 10% trichloracetic acid which precipitated the proteins. The tubes were centrifuged and phosphorus was determined from a 1.5 ml. aliquot of the supernatant.

Color was developed in 15x120 mm. test tubes. 1.5 ml. of the supernatant was pipetted into the tube followed by 2.4 ml. of 0.1 N sodium hydroxide, 1.2 ml. of molybdic acid and 1.2 ml. of stannous chloride in quick succession with thorough shaking. The color intensity developed 23 minutes after the addition of stannous chloride was read in a Coleman Universal Spectrophotometer (model 14) at

600 millimicrons against a blank containing 3.6 ml. distilled water, 1.2 ml. molybdic acid and 1.2 ml. of stannous chloride.

The concentration of phosphorus was calculated from a regression-line equation that was found to describe the calibration curve obtained by plotting % transmission against mg. of phosphorus per 100 ml.

Note

In the following experimental work much of the data obtained has been treated by statistical analysis. In this thesis the value of "*P*" less than 5% is considered statistically significant. "*P*" less than 1% indicates a highly significant difference.

III. INTESTINAL ALKALINE PHOSPHATASE AND ABSORPTION OF LIPIDS IN THE RAT

1. Introduction

From a review of the literature it appears that there are several pathways for the absorption of lipids. Frazer (28) has provided evidence that a portion of the fat enters the systemic blood unchanged by way of the lymphatics. The evidence is not considered to be entirely conclusive. He fed olive oil to rats and was able to observe by microscopic examination that unhydrolyzed particles of fat increased rapidly in systemic blood but not in portal blood. By feeding glycerol and oleic acid he found the reverse to be true. Oleic acid did not produce chylomicronemia. Artom and Swanson (29) have demonstrated that phospholipids may also be absorbed intact.

The general assumption has been that fatty acids are converted to phospholipids in the intestinal mucosa since this fraction of the intestinal lymph has been found to increase after the ingestion of fat (30). However, it has been shown that this mechanism is not obligatory. Zilversmit, Chaikoff and Entenman (31) fed various fats and radioactive phosphorus to animals and found no increase in the amount or turnover in the phospholipids of the mucosa or villi of the small intestine. Verzar and Laszt (32) worked with dogs and

observed that oleic acid was not absorbed _in vitro_ until taurocholic acid was added. Similar experiments on man indicated that bile acids were not necessary for the absorption of fat from the iliac loop (33).

The effect of choline on phospholipid synthesis in the small intestine has been studied by Artom and Cornatzer (34). Choline was found to facilitate phospholipid synthesis in the gut. In this respect Frazer (35) has shown that supplements of choline added to olive oil cleared the intestinal cells of fat more rapidly in the rat.

The observation that labelled fats are incorporated into phospholipid molecules during their absorption led Sinclair (14) to assume that phosphorylation is an obligate stage in fat absorption. This theory is supported by the frequently confirmed work of Flock _et al_ (36) that the phospholipid content of intestinal or thoracic duct lymph is increased during fat absorption. The results of Zilversmit _et al_ (31) however, cast some doubt upon the theory of phosphorylation of fats during absorption.

It has been established that the lipid portion of the diet is effective in elevating serum phosphatase of fasted rats (19)(37). The effect of ingestion of fatty acids upon intestinal alkaline phosphatase of fasted rats is the subject of the present investigation.

2. Experimental

Previous work in this department (23) has indicated that fatty acids, incorporated in a casein carrier and fed to fasting male rats, produced after ingestion an elevation in the intestinal alkaline ohosphatase activity. To confirm this work and extend it, a series of experiments was designed to test the effects of feeding various fatty acids upon intestinal alkaline phosphatase, as well as the effect of choline supplementation.

Oleic acid, an 18-carbon unsaturated fatty acid, and stearic acid, an 18-carbon saturated fatty acid, were incorporated into casein by mixing in a mortar so that a 5 gram portion of the diet contained 0.00035 gm-mole of fatty acid. The concentration of fatty acid is based upon the experiments of Frazer (35) mentioned in the introduction above. Where the effect of choline supplementation was to be studied, choline chloride was added to the diet at two concentrations, 5 mg./30 gm. diet and 10 mg./30 gm. diet.

Five groups of mature, male rats which weighed 250 to 350 grams each were selected, housed in separate cages, were given water to drink ad libitum, and were subjected to a four-day fast. On the fourth day of fasting, five-gram portions of each diet was offered to the animals at 4:30 P.M. 24 hours later the diet was completely consumed and the animals were sacrificed by

decapitation. The intestines were removed and prepared for assay as described in Section II. The results of this experiment are presented in Table I.

In an identical experiment the effect of the fatty acids upon the serum enzyme after 24 hours was determined. The effect of two levels of choline supplementation in the diet is shown in Table II.

From the data in the two tables it was noted that oleic acid produced a highly significant elevation in alkaline phosphatase in the intestine and in the serum. By contrast, stearic acid appeared to have a slight but statistically insignificant effect upon the enzymes as compared with casein controls. In addition, supplements of choline in the diet produced a diminution in the intestinal and serum enzyme response to oleic acid. The effect was significant in the serum and probably significant in the intestine. Choline supplements to stearic acid in the diet were without effect. This present finding has some support in the work of Madsen (38) who found that supplements of choline added to a basal diet of Purina Fox checkers produced a lowering of 20% in serum phosphatase of male adult rats in spite of an increase in body weight during the experimental period.

In order to establish firmly the effect of choline supplementation upon intestinal alkaline phosphatase, an experiment was designed to determine the optimum

TABLE I

EFFECT OF FEEDING CASEIN, FATTY ACIDS, AND CHOLINE SUPPLEMENTS UPON INTESTINAL ALKALINE PHOSPHATASE OF FASTED RATS

No. of Rats	Diet	Alkaline Phosphatase (units/100 gm. wet wt.)
4	A. Casein	9,980±525 *
4	B. Stearic Acid	12,700±1,660
4	C. Stearic Acid + Choline (10mg)	12,200±1,300
4	D. Oleic Acid	18,200±2,000
4	E. Oleic Acid + Choline (10 mg)	14,600±1,330

* Standard error of the mean.

Highly significant difference exists between diets A and D. Value of "P" for diets D and E is 5%.

TABLE II

EFFECT OF CASEIN, FATTY ACIDS, AND CHOLINE SUPPLEMENTS UPON SERUM ALKALINE PHOSPHATASE OF FASTED RATS

No. of Rats	Diet	Alkaline Phosphatase (units/100 ml.)
4	A. Casein	35.10±1.8 *
4	B. Stearic Acid	45.30±4.4
4	C. Stearic Acid + Choline (5mg)	44.50±6.2
4	D. Stearic Acid + Choline (10 mg)	45.30±1.3
4	E. Oleic Acid	60.10±5.9
4	F. Oleic Acid + Choline (5 mg)	40.40±5.0
4	G. Oleic Acid + Choline (10 mg)	38.70±2.3

* Standard error of the mean.

Highly significant difference exists between diets A and E. Values for diets F and G are significantly lowered from the value for diet E.

time for killing the animals. The number of diets was limited to three: a casein control, oleic acid plus casein, and oleic acid plus casein supplemented with choline.

Three groups of animals were selected and treated exactly as in the previous experiments. The concentration of fatty acid in the diet was the same. Choline was supplemented at 10 mg. per 30 gm. diet. Fasting values were obtained from animals sacrificed at 4:30 P.M. From then on rats on each diet were sacrificed at 6, 12, 18 and 24 hours. Serum and intestinal enzyme levels were estimated in the usual way. The results are presented in Table III.

From this table a number of observations can be made. Firstly, under the conditions of this experiment, the enzyme response in the intestine reached a peak after 6 hours on each diet. Secondly, the peaks in serum phosphatase responses occurred after a lag period of 6 hours. Thirdly, it was noted that casein, which was considered to be without effect by previous workers (20)(23), produced a pronounce elevation of the intestine and serum phosphatases of fasting rats. This finding necessitated a search for a more suitable carrier. Wheat gluten was finally chosen (cf. Section V). It was readily eaten by rats and appeared not to affect fasting enzyme levels. The question of intestinal alkaline phosphatase response to the ingestion of fatty acids was then re-examined.

Ten groups of mature, male rats were chosen and treated as in the previous experiments. A variety of fatty acids was incorporated into wheat gluten in a concentration of 0.00035 gm.-mole per five grams of diet. The study of the effect of choline in the diet was repeated. At 4:30 P.M. on the fourth day of fasting, 5 gram portions of each diet were offered to the animals. After 6 hours, the consumptions were checked, the animals were sacrificed by decapitation, and the tissues were removed in the usual way. Intestinal alkaline phosphatase was determined and the data are presented in Table IV.

Again the elevating effect of oleic acid was noted. as well, the low responses due to stearic acid and choline supplementation reoccurred. A better comparison of the effects of the various fatty acids upon intestinal alkaline phosphatase can be seen from Table V. In this table there appears to be an inverse relationship between chain length and degree of enzyme response to the feeding of saturated fatty acids. The introduction of a double bond in the molecule of a fatty acid appears to be associated with increased phosphatase activity.

In the course of a search for a suitable carrier for the fatty acids bran was used but was discarded because it was found to contain 8% fat. The nature of the fat in the product was undetermined. As well, the amount of choline present was not known. The bran was used as a control diet.

TABLE III

EFFECT OF FEEDING CASEIN, OLEIC ACID, OLEIC ACID AND CHOLINE ON THE INTESTINAL AND SERUM ALKALINE PHOSPHATASE OF FASTED RATS AT 6, 12, 18, AND 24 HRS.

Time	No. of Rats	Diet	Alkaline Phosphatase Intestine (units/100 gm)	Alkaline Phosphatase Serum (units/100 ml)
0 Hours	3	Fasting	6,540±756 *	37.70±1.1 (100)
6 "	4	Casein	19,500±1,730	63.60±10.1 (169)
	4	Oleic Acid	24,000±2,000	76.00±4.8 (202)
	4	Oleic Acid+ Choline	18,000±3,390	58.90±2.4 (157)
12 "	4	Casein	13,100±1,470	67.50±13.5 (179)
	4	Oleic Acid	15,600±1,330	90.20±1.1 (240)
	4	Oleic Acid + Choline	13,200±583	83.80±3.6 (223)
18 "	4	Casein	9,540±740	62.30±6.7 (165)
	4	Oleic Acid	11,500±2,180	59.70±1.7 (159)
	4	Oleic Acid + Choline	8,400±1,260	56.10±3.4 (149)

* Standard error of the mean.

(Table continued)

TABLE III (Continued)

Time	No. of Rats	Diet	Alkaline Phosphatase	
			Intestine (units/100 gm)	Serum (units/100 ml)
24 Hours	4	Casein	11,000±1,930 *	39.60±1.1 (105)
	4	Oleic Acid	9,940±2,170	61.00±2.4 (162)
	4	Oleic Acid + Choline	8,340±1,100	57.60±8.6 (153)

* Standard error of the mean.

Figures in brackets indicate per centage of fasting values.

TABLE IV

EFFECT OF FEEDING VARIOUS FATTY ACIDS INCORPORATED IN WHEAT GLUTEN UPON INTESTINAL ALKALINE PHOSPHATASE LEVELS OF FASTED RATS

No. of Rats	Diet	Alkaline Phosphatase (units/100 gm)
7	Butyric Acid	7,060±532 *
7	Lauric Acid	6,730±506
4	Control	4,860±392
7	Palmitic Acid	6,780±415
7	Stearic Acid	5,830±792
6	Control	5,000±469
12	Oleic Acid	8,380±685
8	Oleic Acid + Choline	3,510±436
3	Choline	5,500±1,700
11	Control	4,450±485

* Standard error of the mean.

TABLE V

STATISTICAL ANALYSIS

Fatty Acid	Chain Length	Double Bonds	"t"	"P"
Butyric	C_4	Saturated	3.326	< 0.01
Lauric	C_{12}	"	2.919	< 0.02
Palmitic	C_{16}	"	2.834	< 0.02
Stearic	C_{18}	"	0.928	> 0.10
Oleic	C_{18}	One	4.827	< 0.01
Oleic *	C_{18}	One	3.927	< 0.01
Oleic + Choline	C_{18}	One	1.443	> 0.10

* Oleic acid in this experiment was incorporated in casein at the same concentration. Time of experiment was 24 hours.

Column of "t" values indicates degree of response against a control group.

Oleic acid was added in the usual concentration and fed to fasted rats. Choline chloride, emulsified with oleic acid, was also added to the bran to provide a third diet. Mature, male rats were used in this experiment and were treated the same as in previous experiments. After 6 hours the intestines were removed for enzyme assay. The results obtained are presented in Table VI. The values for the control animals are of the order usually obtained with fasting animals. The addition of oleic acid to the bran produced a statistically significant lowering of the intestinal enzyme. Choline emulsified with oleic acid was able to produce a further significant reduction of the enzyme. The experiment differs from the previous ones in that the control diet contains an undetermined type of fat. The suggestion might be made that phospholipid synthesis is favored in the gut by the presence of fat, fatty acid, and choline, with a concomitant decrease in intestinal phosphatase activity. This explanation has some support in the work of Weil and Russell (37) who found that lecithin in the diet was unable to elevate phosphatase levels of fasting rats.

EFFECT OF FEEDING BRAN, OLEIC ACID, AND SUPPLEMENTS OF CHOLINE UPON INTESTINAL ALKALINE PHOSPHATASE OF FASTED RATS

No. of Rats	Diet	Alkaline Phosphatase (units/100 gm)
6	A. Bran	5,060±343 *
7	B. Oleic Acid	3,950±135
8	C. Oleic Acid + Choline	3,300±205

* Standard error of the mean.

Levels of significance:

A vs. B, $t=2.856$; $P < 0.02$

A vs. C, $t=4.393$; $P < 0.01$

B vs. C, $t=2.313$; $P < 0.05$.

3. Discussion

From an examination of Tables IV and V it is seen that a number of fatty acids when fed to fasted rats are able tp produce an elevation in intestinal alkaline phosphatase. Since the intestine is considered to be a major source of the serum alkaline phosphatase (21), these results appear to agree with the conclusions of Weil and Russell (37) that the fatty acid part of fats is the decisive factor influencing serum phosphatase activity. As well, the statistically significant effect of butyric and oleic acids on the intestinal enzyme confirms previous work done in this department (23). The statistically insignificant effect of stearic acid is also confirmed. This may be due to poor absorption of the fatty acid. Bloom _et al_ (40) fed stearic acid to rats and found 35-54% absorbed in 19-23 hours. Table I shows that stearic acid was able to produce a slightly greater effect upon intestinal phosphatase after 24 hours compared with 6 hours but this was found to be statistically insignificant as well. Further examination of Tables IV and V reveals that the effect of saturated fatty acids upon alkaline phosphatase of fasted rats decreases with increase in carbon chain length. Introduction of a double bond into a long-chain saturated fatty acid results inan increased effect upon the intestinal enzyme. This again is in agreement with the work of Weil and Russell (37)

who found that only unsaturated fatty acids were able to elevated serum alkaline phosphatase of fasted rats. However, they were unable to obtain any effect on the enzyme with a number of saturated fatty acids, including butyric acid.

The question of the absorption pathway of fatty acids is controversial. Frazer (28) worked with rats and found that ingested oleic acid did not give rise to chylomicronemia, and therefore reasoned that the absorption pathway of this fatty acid is through the portal system. A number of workers were unable to confirm this view (41-43). Bloom, Chaikoff and Reinhardt (40) used isotopic C^{14} incorporated in fatty acids and were able to recover 84-95% of the absorbed stearic acid in the intestinal lymph of rats. Myristic acid, a 14- carbon saturated fatty acid was recovered in the intestinal lymph to the extent of 59-82%. Lauric acid, a 12-carbon saturated acid was almost completely absorbed and was recovered to the extent of 15-55% in the lymph. 7-19% of the completely absorbed decanoic acid was recovered in the intestinal lymph. The suggestion is made by these workers that for the lower chain fatty acids a portal pathway is indicated. In an earlier paper, these men report (45) that 90% of the labelled fatty acid fed to rats was recovered in the lymph in a non-phospholipid form, i.e., they are carried in the

form of glycerides.

It may be possible to correlate these findings with the data presented in Tables IV and V. Short-chain saturated fatty acids produce an elevation in intestinal alkaline phosphatase of rats. Low recoveries of these acids suggests a portal pathway for absorption (40). Long-chain fatty acids are less effective upon alkaline phosphatase. High recoveries in the intestinal lymph indicate a lymphatic pathway for these acids (44). Finally, the high effect of oleic acid upon the intestinal enzyme may be correlated with the portal pathway for this acid as suggested by Frazer (28). It should be noted however, that Flock and Bollman (46) have demonstrated the presence of high concentrations of alkaline phosphatase in the lymph of rats.

Supplements of choline in the diet appeared to decrease significantly the effect of oleic acid upon the intestinal enzyme of fasted rats (Table IV). Artom and Cornatzer (34) have shown that both fat and choline are involved in phospholipid formation in the intestine. These authors believe choline to be a limiting factor in the formation of phospholipid during the absorption of fat. If this true, choline may be considered to exert a mass action effect favoring formation of phospholipid in the gut. A recent report (47) contains the suggestion that

choline accelerates phospholipid turnover by such a mass action effect. In this respect Weil and Russell (37) found that lecithin in the diet was without effect upon serum alkaline phosphatase of fasted rats. In the present experiments choline in the diet maintained intestinal phosphatase values near the control levels. This finding has support from the work of Madsen (38) in this department. It may be possible to correlate decreased alkaline phosphatase activity of the intestine of rats with accelerated phospholipid turnover.

4. Summary

The effect of ingestion of various fatty acids upon intestinal alkaline phosphatase of fasted rats is presented and discussed. Short-chain saturated fatty acids and a long-chain unsaturated fatty acid were more active in this respect than long-chain saturated fatty acids. Supplements of choline in the diet produced a diminution in alkaline phosphatase. This is suggested as a mass action effect favoring formation of phospholipid in the intestine.

IV. INTESTINAL ALKALINE PHOSPHATASE AND ABSORPTION OF CARBOHYDRATES IN THE RAT

1. Introduction

Sugars are lipoid-insoluble non-electrolytes and therefore could be expected, by their rates of absorption, to follow the diffusion law, where the molecular volume is the determining factor. That this behavior does not obtain normally was indicated by the discovery (48)(49) that hexoses are not absorbed with equal speed, and that pentoses pass even more slowly across a selective barrier than the hexoses. The lower rate of absorption of disaccharides compared with monosaccharides, however, could be correlated with the greater molecular volume and the lower diffusion rate.

Today it is accepted that different sugars are absorbed at different rates from the intestine. The physiologically important hexoses, glucose, galactose, and fructose are known to be more rapidly absorbed than mannose and the pentoses. By the use of different methods, Nagano (50) working on dogs, Cori (51)(52), and Wilbrandt and Laszt (53) working on rats, have obtained the following relative values as an index of the speed of absorption: galactose 115, glucose 100, fructose 44, mannose 33, sorbose 30, xylose 30, arabinose 29.

Verzar (39) has postulated that the rapid absorption of glucose, galactose, and fructose is directly due to the phosphorylation of these hexoses within the mucous membrane of the gut. The less rapidly absorbed sugars were considered not to undergo phosphorylation but to pass through the mucosa by a passive process.

Part of the evidence supporting this hypothesis is the work of Lundsgaard (55) who demonstrated that iodoacetate inhibited the absorption of glucose, galactose, and fructose by interfering with enzymic phosphorylations. No interference in the absorption of xylose or pentose was noted. The acid-soluble organic phosphate fraction of the intestine was considerably increased during absorption of the three hexoses, but not during the slow absorption of xylose, arabinose, or mannose (56)(57). Iodoacetate was shown to prevent an increase in acid-soluble phosphate otherwise obtained during absorption of the physiologically important hexoses (58).

Various workers (59-60) have criticized the use of iodoacetate on the grounds that monoiodoacetic acid is not a specific inhibitor of phosphorylation but a general systemic poison inihibiting indifferently the absorption of glucose, xylose, and NaCl.

The objections to these experiments were overcome to some extent by the use of the plant glucoside phlorrhizin which attacks an oxidative process in the cell with which

phosphorylation is coupled (61). The respiratory rate of the cell is not affected. Nakasawa (62)showed the agent to be capable of markedly and reversibly inhibiting the absorption of the physiologically important hexoses by the small intestine. Beck (63) used phlorrhizin and was led to conclude that the simultaneous inhibition of glucose absorption is due to interference with glucose phosphorylation.

Still, this evidence has been seriously questioned by Hestrin-Lerner and Shapiro (59) since it was found that the concentration of the glucoside used in the above experiments was sufficient to cause complete inhibition of glucose absorption by the kidney, but had no measurable effect on glucose phosphorylation (60). However, Verzar's hypothesis is now generally accepted and is also regarded as applicable to the absorption of glucose in all types of animals.

Direct evidence of the phosphorylation of sugars is provided by the experiments of Hele (66). He has demonstrated that the phosphorylation-rate ratios of the sugars are very similar to their absorption-rate ratios. By a study of the hexokinase reaction, the dephosphorylation of hexose phosphates, and adenosine triphosphatase activity in dispersions of intestinal mucosa, different sugars were found to become phosphorylated at different rates.

The functional importance of serum phosphatase has been indicated by the work of Bodansky (67) on dogs. Prolonged fasting was found to reduce greatly the serum phosphatase activity of young dogs and an increase in activity was obtained only after ingestion of carbohydrates. This was considered due to increased functional activity with transitory hexose phosphate formation and production of phosphatase by the intestinal mucosa, which is supported by the works of Lundsgaard(10) and Verzar (67)(68) on carbohydrate absorption. Yet, subsequent investigations have not been able to substantiate these findings. Weil and Russell (37) followed by Taylor, Madsen and Tuba (69) showed that only the lipid fraction of diets was active in restoring low fasting serum phosphatase levels in rats to normal values. High levels of alkaline phosphatase were found in the intestinal lymph following the feeding of fats to rats (46). Other foodstuffs did not produce this response. Lawrie and Yudkin (71) in studies on biochemical adaptation noted that high concentrations of sucrose in the diet produced a reduction in the amount of alkaline phosphatase in the intestinal mucosa of rats compared with a high fat, high protein, and a basal diet.

After it was established that certain fatty acids were able to produce an intestinal alkaline phosphatase response (cf. Section III above), presumably by a phosph-

orylation-dephosphorylation mechanism, it was suggested to this author to study the effect of various carbohydrates on the intestinal enzyme.

2. Experimental

It was first necessary to study carbohydrate absorption interms of phosphatase activity over a number of hours in order to determine the optimum time for sacrificing the animals. For this preliminary experiment two carbohydrates were chosen, glucose and xylose, which would be representative generally of hexoses and pentoses respectively in their absorption patterns.

Male albino rats which weighed 250 to 350 grams each were chosen. They were prepared as indicated in Section II except that the fasting period was extended to 5 days. A five-day fast was necessary to ensure adequate and consistent consumption of the diets. The monosaccharides were incorporated in cellulose at 30% level by mixing. At 4:30 P.M. of the fifth day the animals received 5 grams of each diet. The control animals received cellulose alone. It had been previously noticed that cellulose exerts no effect upon intestinal alkaline phosphatase and apparently passes through unchanged. In this preliminary experiment starved animals were used as additional controls. Fasting values were obtained from animals sacrificed at 4:30 P.M. Following this, rats were sacrificed after 2, 4, 6, 7, 9, and 11 hours on diet. The first ten cm. of intestine were removed and treated as described in Section II. Phosphatase determinations were done and the results summarized in Table VII.

TABLE VII

EFFECT OF CELLULOSE, GLUCOSE, AND XYLOSE AT TWO HOUR INTERVALS ON INTESTINAL ALKALINE PHOSPHATASE OF FASTED RATS. Monosaccharide consumed, (C) in grams; Alkaline Phosphatase, (P) in units/100 gm.

Time on Diet	Starved	Cellulose	Glucose		Xylose	
	P	P	P	C	P	C
0 Hours	6,780	-	-	-	-	-
2 "	5,740	6,460	8,750	1.35	11,100	1.41
4 "	-	5,780	10,300	1.44	13,900	1.11
6 "	5,740	6,900	13,100	1.50	19,800	1.26
7 "	6,300	7,530	10,500	1.08	16,100	0.84
9 "	6,740	7,620	8,110	1.20	10,700	1.05
11 "	4,110	4,390	4,760	1.44	11,100	1.41

All values are means for two animals.

The magnitude of the response to the feeding of xylose in this preliminary experiment was unexpected and has never been obtained since. The reason for this unusual behavior of the pentose is unknown. However, it was reasoned that, if pentoses are active in their effect upon the intestinal enzyme, the optimum time was therefore indicated. Cellulose was again shown to produce no significant elevation above fasting levels.

As the data of Table VII seemed to indicate that sugars fed to fasted rats produce increases in the intestinal alkaline phosphatase activity, it was then decided to study the effects of a variety of carbohydrates in the diet upon the enzyme. The optimum time for killing was established at 6 hours on diet.

Seven groups of animals were selected which contained five to seven rats in each group. They weighed between 250 to 350 grams each, and were treated as in the preliminary experiment. Each carbohydrate was incorporated in cellulose at a 30% level. The D- or natural isomers were used in every case. On the fifth day of fasting 5 grams of each diet was placed before the animals. After 6 hours on diet, consumptions were checked for uniformity, and the animals were sacrificed by decapitation. Phosphatase levels were determined after treatment of the guts as in the preliminary experiment. The data are presented in Tables VIII and IX.

In an effort to determine whether the enzyme response to carbohydrate ingestion is reflected in the serum after 6 hours, blood from the decapitated animals was collected and serum phosphatase estimations were carried out on the following day.

TABLE VIII

EFFECT OF INGESTION OF VARIOUS CARBOHYDRATES UPON INTESTINAL ALKALINE PHOSPHATASE OF FASTED RATS

No. of Rats	Diet	Alkaline Phosphatase (units/100 gm.)
7	Cellulose	3,970±514 *
6	Sucrose	5,140±300
6	D-Glucose	10,200±652
5	D-Galactose	6,600±475
7	D-Fructose	6,480±726
7	D-Mannose	7,790±1,250
6	D-Arabinose	4,650±631
5	D-Xylose	5,880±753

* Standard error of the mean.

TABLE IX

STATISTICAL ANALYSIS

Carbohydrate	Activity (units/100 gm)	"t"	"P"
1. D-Glucose	10,200	5.640	Less than 1%
2. D-Galactose	6,600	3.752	"
3. D-Mannose	7,790	2.836	Less than 2%
4. D-Fructose	6,480	2.823	"
5. D-Xylose	5,880	2.089.	Greater than 5%
6. Sucrose	5,140	1.953	"
7. D-Arabinose	4,650	0.829	"
8. Cellulose	3,970	-	-

The data summarized in Table X indicate fasting serum alkaline phosphatase values after 6 hours on diet in every case. This serves to strengthen the idea presented in the section on lipid absorption (Section III) that serum enzyme responses reflect those of the intestine after a lag period of about 6 hours, under the conditions of these experiments.

The unexpected low response of the intestinal enzyme to the sucrose diet presented the possibility that sucrase activity in the gut is diminished in the fasted animals in the region where intestinal absorption is measured, and the sucrose in the diet was passing through unchanged. To investigate this point, four normal, male rats, maintained on checkers, and four animals fasted five days were sacrificed, the intestines removed and immediately homogenized. Sucrase determinations were done by a modification of the method of Sumner and Howell (70). A calibration curve for invert sugar was constructed by using 0.1% D-glucose in saturated benzoic acid as a standard (Table XI). Reduction was estimated by the dinitrosalicylic acid method (70).

The first ten cm. of intestine were removed, homogenized in cold, distilled water, and made up to 25 ml. Five ml. of 6.5% sucrose buffered at pH 6.0 by phosphate was placed in a water bath at 37° C. One ml. of the enzyme was added, mixed, and incubation was allowed to proceed for 1 hour. The invert sugar produced was estimated by the dinitrosalicylic acid method. Readings were taken at 560 millimicrons against a sucrose blank on the Coleman

TABLE X

SERUM ALKALINE PHOSPHATASE RESPONSE OF FASTED RATS TO VARIOUS CARBOHYDRATES AFTER 6 HOURS ON DIET

No. of Rats	Diet	Alkaline Phosphatase (units/100 ml.)
5	Cellulose	37.20±2.1 *
5	Sucrose	33.70±1.7
5	D-Glucose	36.20±1.7
4	D-Galactose	38.10±1.5
5	D-Fructose	36.80±1.4
2	D-Mannose	35.10±3.2
4	D-Arabinose	34.60±2.3
4	D-Xylose	37.10±2.1

* Standard error of the mean.

TABLE XI

CALIBRATION CURVE FOR ESTIMATION OF SUCRASE ACTIVITY

Mg. sugar/ml. enzyme	% Transmittance				
	560	570	580	590	600
			(millimicrons)		
0	100	100	100	100	100
0.05	100	100	100	100	100
0.10	95.4	96.2	96.8	97.7	98.3
0.50	54.6	59.6	64.5	69.6	74.3
1.00	29.0	34.6	40.4	46.5	53.6

spectrophotometer. The results are presented in Table XII.

It is seen that, although the sucrase activity is low in the intestinal mucosa of rats, a five-day fast does not appear to alter the enzyme level significantly. Presumably, the enzyme is capable of acting upon sucrose in the diet to the same extent as in the normal animal.

TABLE XII

SUCRASE ACTIVITY IN INTESTINAL HOMOGENATES OF NORMAL AND FASTED RATS

	Mg. invert sugar produced/gm. tissue	
Rat	Normal	Fasted
1.	8.63	8.13
2.	7.35	6.43
3.	6.73	6.82
4.	4.89	6.48
Mean	6.90	6.48

3. Discussion

From the data presented in Tables VIII and IX several observations can be made. Firstly, the results presented appear to support the hypothesis of Verzar (39) to the extent that an enzymic reaction is shown to be involved in the active absorption of certain sugars from the intestine of the rat. Secondly, it can be seen that hexoses, in contrast to pentoses, when fed to fasting rats, were able to elevate significantly intestinal alkaline phosphatase compared to control animals fed cellulose. This finding lends additional support to the concept of rapid absorption of the physiologically important sugars (39). It is not implied, however, that absorption of pentoses does not involve enzymic processes. It has been demonstrated in the cat (66) that xylose is as rapidly absorbed from the intestine as is glucose. Hele (66) has shown that the rate of the hexokinase reaction depends upon the concentration of sugar and ATP. He was able to demonstrate *in vitro* that, with increased concentrations of mannose, the rate of phosphorylation of this sugar approached that of glucose.

It is emphasized that the results presented in Tables VIII and IX do not indicate the actual mechanism of absorption of sugars from the gut. More direct evidence in this respect is provided by Hele (66).

The effect of sucrose upon alkaline phosphatase of rats has been noted by other workers. Weil and Russell (37) fed sucrose to 5-weeks old rats and found no elevation in serum phosphatase. Lawrie and Yudkin (71) in studies on biochemical adaptation found that the presence of sucrose in the diet led to a reduction in intestinal alkaline phosphatase of rats. In the present experiment, hydrolysis of sucrose in the gut is presumed on the basis of the data in Table XII. Hele (66) has provided evidence that satisfactorily explains the sucrose effect. He tested a number of sugar mixtures and found that all mixtures examined produced a depression in the rate of the hexokinase reaction of intestinal homogenates of rats. The suggestion was made by this author that competition between sugars for hexokinase occurs in mixtures, somewhat along the lines indicated by Slein, Cori, and Cori (72).

4. Summary

The effect of ingestion of various carbohydrates on intestinal alkaline phosphatase of fasted rats is presented and discussed. Each hexose tested was able to elevate significantly the fasting levels of the intestinal enzyme, and the effect was greatest with glucose. By contrast, pentoses and sucrose were without effect. In the case of sucrose, this was considered to be due to competition for hexokinase between the hydrolytic products of the disaccharide.

V. INTESTINAL ALKALINE PHOSPHATASE OF RATS AND THE EFFECT OF DIETARY PROTEIN

1. Introduction

A highly significant effect of casein in the diet upon intestinal alkaline phosphatase of fasted rats was noted during the work on lipids (cf. Table III, Section III). This finding raised the question whether one or several amino acids liberated by protein digestion were actively absorbed in the gut by a system involving alkaline phosphatase. Previously, Tuba et al (20) fed diets containing graded amounts of casein to weanling male albino rats with animals deprived of protein serving as controls. A highly significant correlation was obtained between daily food consumption and serum alkaline phosphatase activity which they attributed to the fat present in the diet (3%). No effect upon the enzyme was attributed to casein. However, the report of Lawrie and Yudkin (71) supports the present finding. 70% casein in the diet was found by these workers to produce an elevation in the intestinal phosphatase activity of rats of the same order as did 70% fat. Animals fed a basal diet containing 15% casein and 15% fat served as controls.

The effect of dietary casein upon the intestinal enzyme was considered from the point of view that the protein contains orthophosphoric acid as a prosthetic group. Casein of milk and vitellin of egg yolk are the two most important

phosphoproteins. For casein, it is known that orthophosphoric acid is esterified to the -OH group of serine (73).

Several workers report that phosphatases have no action on casein (74)(75) although the phosphopeptone resulting from tryptic hydrolysis of casein has been shown to be attacked by kidney and bone phosphatases (76)(77). This suggests that possibly serine phosphate is not available as a substrate for phosphatase within the spatial configuration of the casein molecule.

Casein is known to be a mixture of two proteins: α-casein and β-casein, which differ in solubility (78), phosphorus content (79), and electrophoretic behavior (80). Perlmann(81) has shown that differences also exist in the action of phosphatases on the two fractions. α-casein is readily dephosphorylated at pH 5.6 to 6.6 by prostatic phosphatase. The enzyme has no effect upon β-casein. In this respect, it is interesting that evidence has been presented which indicates a specific phosphoprotein phosphatase in frog' eggs (82), ox-spleen (83), and rat spleen (84). The enzyme liberates part of the phosphoric acid from phosphoproteins and phosphopeptone, but not from certain phosphopeptides, diphenyl phosphate, or β-glycerophosphate.

The following experiments were designed to test the effect of phosphoproteins in the diet upon intestinal alkaline phosphatase of fasted rats. Other proteins were

fed for comparison. Any effect obtained with casein and vitellin could presumably be attributed to serine phosphate undergoing dephosphorylation, particularly if the other proteins were without effect.

2. Experimental

It was decided to follow again, the effect of feeding casein on intestinal alkaline phosphatase over a number of hours (cf. Table III, Section III). As well, lactalbumin was chosen for comparison. This was in the nature of a preliminary experiment.

Three groups of mature male rats were fasted for four days, given water to drink ad libitum during this period, and then were placed on diet. One group of animals received 5 grams of casein. A second group received 5 grams of lactalbumin, while a third group received cellulose powder. The animals were sacrificed by decapitation at intervals after being placed on diet. Fasting values were obtained from animals at 4:30 P.M. Phosphatase determinations were done as described in Section II. The results are summarized in Table XIII.

The effect of feeding casein upon the intestinal enzyme of fasted rats was confirmed by this preliminary experiment in which a significant elevation was again obtained after 6 hours compared with the control animals. Lactalbumin in the diet was without effect. Following this, a number of proteins were selected as test diets. Mature male rats were chosen and treated as in the preliminary experiment. They were divided into seventeen groups. Each animal in a group received 5 grams of a respective protein.

Cellulose served as a control diet.

The animals were allowed to remain on diet for 6 hours. The consumptions were checked and the animals sacrificed. Intestinal enzyme estimations were done and the results are summarized in Table XIV.

It is evident from this table that only the two phosphoproteins, casein and vitellin, were able to elevate the intestinal enzyme above control values.

TABLE XIII

EFFECT OF CASEIN, LACTALBUMIN, AND CELLULOSE ON INTESTINAL ALKALINE PHOSPHATASE OF FASTED RATS OVER 15 HOURS

Diet	Time in Hours			
	0	3	6	15
Cellulose	3,080 *	3,260	2,880	3,000
Casein	-	2,410	7,200	7,280
Lactalbumin	-	2,810	3,980	2,900

* Values are means of two animals expressed as units/100 gm. wet tissue.

EFFECT OF VARIOUS DIETARY PROTEINS ON INTESTINAL ALKALINE PHOSPHATASE OF FASTED RATS

Set No.	No. of Rats	Diet	Alkaline Phosphatase (units/100 gm.)	"t"	"P"
1.	7	Casein	6,180±614 **	3.823	<1%
	5	Control	3,470±350		
2.	18	Lactalbumin	4,580±303	-	-
	12	Control	4,270±357		
3.	10	Zein	4,540±347	-	-
	9	Egg Albumin	3,670±401	-	-
	11	Control	4,050±355		
4.	4	Gelatin	5,540±541	-	-
	4	Control	4,740±439		
5.	6	Wheat Gluten	3,730±541	-	-
	8	Control	3,420±438		
6.	5	Vitellin	6,120±518	3.933	<1%
	9	Control	3,900±223		
7. *	4	Vitellin	13,200±1,180	3.118	<2%
	6	Control	9,340±384		
8.	4	Bran	5,750±1,160	-	-
	4	Control	4,890±155		

** Standard error of the mean.

* Animals in this set exhibited high control and test activities. Reason unknown.

3. Discussion

It is possible to suggest on the basis of the data in Table XIV that a phosphorylated amino acid may conceivably be absorbed from the intestine by an active process involving alkaline phosphatase. It does not seem probable that many amino acids are absorbed by such a process. Presumably, some phosphorylation mechanism by hexokinase would be required. Active transport of amino acids against a concentration gradient has been demonstrated by Agar, Hird and Sidhu (85) who used isolated loops of rat intestine. As well, it has been shown (86) that amino acids inhibit the transfer of other amino acids when fed in mixtures. Preliminary experiments in this department indicate that certain amino acids fed to fasted rats in cellulose plus sucrose as a carrier produce an effect upon the intestinal phosphatase. In particular, serine when fed to fasted rats was found to produce an elevation of the enzyme (87).

4. Summary

The effect of ingestion of a variety of proteins upon intestinal alkaline phosphatase of fasted rats is presented and discussed. Casein, and vitellin were shown to elevate significantly the fasting levels of the enzyme. The possibility that this effect may be due to the active absorption of a phosphorylated amino acid involving phosphatase is mentioned. The remaining proteins tested were without effect.

VI. CYTOCHEMICAL STUDIES: DISTRIBUTION OF ALKALINE PHOSPHATASE IN CELLS OF THE RAT INTESTINE

1. Introduction

Attempts to apply analytical methods to the study of cell structures by isolation of known cellular components date back at least to the work of Warburg (88) in 1913. In that year he was able to segregate cytoplasmic granules from cell-free extracts of guinea-pig liver and show that these were responsible for most of the oxygen uptake. In 1934 Bensley and Hoerr (89) described the isolation of mitochonfria by the method of differential centrifugation. Since this time, the method has been improved and extended to the complete fractionation of the cell. Comprehensive fractionation procedure have been described (90-92) that enable the separation of tissues into four fractions: a nuclear fraction, a mitochondrial fraction, a submicroscopic-particle or microsomal fraction, and a supernatant or soluble fraction. The last three fractions contain essentially the components of the cytoplasm.

The procedure of cell fractionation is considered by many workers to be a much more versatile method than others available in the field of cytochemistry (44). By this method cell components can be obtained in practically unlimited amounts, thus enabling full use of biochemical methods. As well, the whole range of particle and molecular sizes can be investigated since centrifuges can provide

centrifugal fields of very high strength.

Essentially, the method consists of the mechanical rupture of tissue cells in a suitable medium followed by isolation of the various cellular components by differential centrifugation. It is understood that the cell is not a continuum of protoplasm but consists of a number of discrete structures which are distinct in size, chemical composition, and functions. Next in size to the nuclei are the mitochondria, which may be recognized by their location in the cell and by certain staining reactions. They are filamentous or rod-like bodies and are the site of many important oxidative reactions in the cell. The microsomes are complex submicroscopic elements, the origin of which is not yet settled. The discrete structures of the cell can therefore be separated from each other because of their differences in size and specific gravity.

Claude (94) states that, following disruption of the cellular membrane, nuclei and debris as well as unbroken cells can be removed by low-speed centrifugation. The elements of the cytoplasm can then be routinely fractionated at appropriate speeds to give (a) a mitochondrial fraction consisting of particles 0.5 to 2.0 µ in diameter, (b) a microsome fraction consisting of particles approximately 50 to 200 mµ in diameter, (c) a supernatant fraction containing the soluble elements of the cell of low molecular weight and less than 50 mµ in diameter.

Claude emphasizes that the division of the cytoplasmic extract is not based on size differences alone since each fraction possesses characteristic physical, chemical, and biochemical properties. Also, it is not implied that each fraction is homogeneous, i.e., the statement is not made that each particle in a certain fraction is identical in these properties to another particle in the same fraction. In this respect, Paigen (95), working with rat liver, and Kuff and Schneider (96), working with mouse liver, have indicated a biochemical heterogeneity among mitochondria. Paigen has presented evidence of the existence of at least three types of mitochondria that differ from each other in their ultraviolet absorption spectra and their contents of cytochrome oxidase, succinic oxidase, D-amino acid oxidase, and various phosphorus-containing compounds.

One drawback to the method of cell fractionation is the danger of artifacts being formed during the procedure. This may consist of redistribution of activities, adsorption, morphological alterations, etc. (44). However, Schneider and Hogeboom (93) consider the isolation of a cell component in a morphological and cytologically unaltered form as a sufficient guarantee of the integrity of the component. In this respect, they point out that the structural components of the cell, upon disruption of the membrane, are released into an abnormal environment. The choice of

medium is then of considerable importance. These workers have demonstrated that electrolytes in the medium caused aggregation of mitochondria preventing a satisfactory separation (91). Aggregation of cellular particles was found not to occur in 0.88 M and 0.25 M sucrose. They found that 0.88 M sucrose was able to preserve the normal morphological characteristics of the mitochondria. However, very high centrifugal speeds were necessary when they used this medium. 0.25 M sucrose produced a sphering of the mitochondria but had the advantage that much lower speeds were required for centrifugation. A comparison of the two media by Hogeboom and Schneider indicated no essential differences as far as total nitrogen and certain enzymes were concerned. Isotonic sucrose is considered by these authors to be the medium of choice.

The method used for cell disruption is one factor that affects the yield in cell fractionation studies (44). Various reports state that the Waring Blendor is unsatisfactory in many respects (44,97). Schneider (98) considers the use of the Potter-Elvehjem homogenizer (24) as the best available method.

The object of the present study was to determine the localization of alkaline phosphatase in the intestinal cells of the albino rat. The intestinal cells were completely fractionated and each fraction was assayed in order to

satisfy certain criteria that must be met before a biochemical property can be ascribed to a given structural component of the cell (93).

2. Experimental

Adult male albino rats which weighed 200 to 250 grams were employed. They were maintained on a diet of Purina Fox checkers and were fasted overnight prior to experiment. In the morning the rats were killed by decapitation. The first ten cm. of intestine from the pylorus were excized and placed in a small beaker cooled by an ice-bath. The piece of gut was then washed out by a stream of cold isotonic sucrose solution from a water-bottle, dried by light blotting on filter paper, and was weighed to the nearest one hundredth of a gram. This portion of intestine was then homogenized in ice-cold 0.25 M sucrose by means of a Potter-Elvehjem glass homogenizer and was kept cool by an ice-bath. The homogenate was then made up to 25 ml. volume with isotonic sucrose to contain 0.02-0.03 gm. of tissue per ml.

The fractionation procedure which was followed is essentially the one described by Schneider (98). The centrifugal speeds used in this experiment were slightly higher in order to ensure good sedimentation of the various components of the cell. They were calculated from the following equation (98):

$$f = \frac{w^2 r}{980} = \frac{s^2 r}{89,500}$$

where w = angular velocity
r = radial distance in cm.
s = revolutions per min.
f = centrifugal force in gravitational units, *g*.

All centrifugations were carried out in an International refrigerated centrifuge (model PR-1) at 2-3° C; gravitational forces were calculated for the centre of the tube. For the low speed head the radial distance was 14 cm., for the high speed head, 6 cm. The fractions collected were stored at 2-3° C. and enzyme determinations were done in the morning after centrifugation was complete. All glassware was pre-chilled and packed in crushed ice during use.

A 20 ml. aliquot of the homogenate was taken for fractionation, pipetted into two 15 ml. lusteroid round-bottomed tubes and centrifuged for 10 min. at 650 x *g*. Nuclei, unbroken cells, and debris were sedimented by the first spinning. The supernatant was drawn off and centrifuged at 650 x *g* for 10 min. to remove nuclei completely. The sediments were washed twice by rehomogenizing each time in 3.0 ml. of isotonic sucrose and centrifuging for 10 min. at 650 x *g*. The sediment which remained after the final centrifugation was suspended in isotonic sucrose and labelled the "nuclear fraction". The supernatants obtained from this fraction were combined and subjected to 9,500 x *g* for 10 min. in 50 ml. lusteroid tubes with the multi-speed

attachment and Head No. 291. The sediment was well packed at this speed and appeared as a pellet of brown material at the bottom of the tube surrounded by a fringe of white substance. Very light, poorly-sedimented wisps of material were withdrawn with the supernatant. These are considered by Schneider (98) to be submicroscopic particles. The sedimented material was washed twice by homogenizing in 3 ml. of 0.25 M sucrose and recentrifuging at 9,500 x g. The final sediment was resuspended in isotonic sucrose and labelled the "mitochondrial fraction". The supernatants from the mitochondria were combined and centrifuged for 1 hour at 20,000 x g in order to sediment the submicroscopic particles. The material when packed by centrifugation exhibited a pronounce red color that was translucent when held up to the light. This is considered to be due to the presence of pigments produced by oxidation of unsaturated fats, particularly phospholipids (54). The sediment was washed once by resuspending in 0.25 M sucrose and resedimenting at 20,000 x g. The final sediment was resuspended in isotonic sucrose and labelled the "microsome fraction". The supernatants from this fraction were combined to form the "supernatant fraction".

Microscopic examination of the various fractions was carried out by means of the light microscope using dark field illumination. The "nuclear fraction" was seen to contain nuclei, debris, and a few mitochondria. The ex-

aminations were done on materials that were unfixed and unstained. The "mitochondrial fraction" contained spherical bodies that appeared to be identical to those described by Hogeboom and Schneider (91) as being mitochondria. This fraction appeared entirely homogeneous and well-defined. No contamination by nuclei was noticed. The "microsome fraction" was free from mitochondria. The "supernatant fraction" was optically empty to the light microscope.

The nuclear, mitochondrial, and microsome fractions were made up to 50 ml. with 0.25 M sucrose. The supernatant was made up to 100 ml. Alkaline Phosphatase estimations were done on a 1:100 dilution of these fractions. Total homogenate activity was determined on a 1:250 dilution. Total nitrogen was determined according to the method of Ma and Zuazaga (65).

(i) Fractionation of Intestine of Normal Rats.

The distribution of alkaline phosphatase activity in fractions from the intestines of normal rats was determined. The results of a typical fractionation are presented in Table XV. It will be noted that satisfactory recoveries of phosphatase activity and nitrogen were obtained from the four fractions assayed. The validity of the phosphatase assay is thus indicated. Of interest is the finding that although the enzyme activity appears to be distributed throughout the cell, the major part of the activity in

TABLE XV

DISTRIBUTION OF ALKALINE PHOSPHATASE ACTIVITY AND NITROGEN IN FRACTIONS ISOLATED FROM HOMOGENATES OF RAT INTESTINE IN 0.25 M SUCROSE. RESULTS ON ONE NORMAL MALE RAT

Fraction	Nitrogen (mg)	%	Alkaline Phosphatase (units/gm)	%	Specific Activity (units/mg N)
Homogenate	19.95	100	130.90	100	4.6
Nuclei	5.15	26.9	26.40	20.2	3.7
Mitochondria	1.70	8.9	14.30	10.9	6.0
Microsomes	1.65	8.6	66.40	50.8	28.6
Supernatant	11.70	61.0	31.40	24.0	1.9
Recovery	20.20	101	138.50	106	

the intestinal cell is associated with the microsome fraction. Schneider (98) considers two criteria must be met before an enzyme system can be said to be predominantly associated with a particular cell structure, viz., the major portion of the total enzyme activity must be recovered in the particular cell fraction, and, the specific activity (activity per mg. nitrogen) of this fraction must be several times that of the total. Of course it is also necessary that good recoveries be obtained from the fractions. It is seen from the results recorded in Table XV that these criteria are met.

Five normal male animals were sacrificed, the usual portion of intestine was fractionated and assayed for alkaline phosphatase. The results appear in Table XVI. It seems certain from these data that the enzyme is definitely associate with each cell fraction. The specific activity is the highest in the "microsome fraction". It is also evident from Table XVI that the enzyme recoveries are greater than 100%. It is possible that unbroken cells in the "nuclear fraction" contributed their mitochondrial, microsomal, and supernatant activities. Yet, the low standard error of the mean for this fraction indicates a uniform yield of activity for five animals. Subsequent work on fasted animals suggested the idea that some activator may have fallen into some fraction to produce slightly higher activities (98).

TABLE XVI

DISTRIBUTION OF ALKALINE PHOSPHATASE ACTIVITY AND NITROGEN IN FRACTIONS ISOLATED FROM HOMOGENATES OF RAT INTESTINE IN 0.25 M SUCROSE

Fraction	% Nitrogen	% Alkaline Phosphatase Activity	Specific Activity
Homogenate	100	100	7.1
Nuclei	32.0±5.9 *	17.0±1.6	4.1
Mitochondria	8.7±4.2	11.8±1.1	7.4
Microsomes	13.1±5.1	52.3±1.9	24.5
Supernatant	46.1±5.9	31.4±2.2	6.2
Recovery	98.9±5.2	112 ± 2.1	

All values are means for 5 animals.

* Standard error of the mean.

(ii) Fractionation of Intestines of Fasted Rats.

Mature male rats were fasted for 6 days but had water before them during that period. They were then killed and their intestines fractionated in the same way as the normal animals. Alkaline phosphatase distribution was determined. The results of a single fractionation are recorded in Table XVII. The homogenate activity is of the order usually obtained for fasting rats, i.e., 5,000 to 6,000 units per 100 grams of wet tissue. The recovery of nitrogen was satisfactory which indicated a valid fractionation. The feature of this fractionation is the large recovery obtained from the four fractions. Each has a greater per centage activity with the exception of the microsomes. The specific activity values indicate increased importance of the mitochondrial and the soluble alkaline phosphatase. The results of 6 fractionations are presented in Table XVIII.

The distribution of phosphatase activity from homogenates of starved rat intestines was found to exhibit two patterns on the basis of 6 experimental animals. In set no. 1 two animals displayed a pattern very similar to that of a normal distribution as indicated in the column "Factor". The recoveries are the same as for the normal rat. However, the microsomes appear to have lost part of their activity, which may be due to leakage of the enzyme into the "super-

TABLE XVII

DISTRIBUTION OF ALKALINE PHOSPHATASE ACTIVITY AND NITROGEN IN FRACTIONS ISOLATED FROM HOMOGENATES OF RAT INTESTINE IN 0.25 M SUCROSE: RESULTS ON ONE MALE FASTED 6 DAYS

Fraction	Nitrogen (mg)	%	Alkaline Phosphatase (units/ gm.)	%	Specific Activity (units/mg. N)
Homogenate	13.80	100	63.10	100	2.6
Nuclei	4.60	33.3	15.50	24.3	1.9
Mitochondria	1.10	8.0	16.20	25.7	8.4
Microsomes	1.95	14.1	31.60	50.0	9.2
Supernatant	5.70	41.3	48.30	76.4	4.8
Recovery	13.40	96.7	111.0	176.0	

TABLE XVIII

DISTRIBUTION OF ALKALINE PHOSPHATASE ACTIVITY IN FRACTIONS ISOLATED FROM HOMOGENATES OF INTESTINES IN 0.25 M SUCROSE FROM RATS FASTED FOR 6 DAYS

Set No.	No. of Rats	% Alkaline Phosphatase		Factor (comparison with normal)
1.	2	Homogenate	100	1.0
		Nuclei	18.7	1.1
		Mitochondria	14.0	1.2
		Microsomes	32.7	0.6
		Supernatant	48.6	1.5
		Recovery	114.0	1.0
2.	4	Homogenate	100	1.0
		Nuclei	27.2±1.2 *	1.6
		Mitochondria	20.1±3.0	1.7
		Microsomes	53.3±2.4	1.0
		Supernatant	66.3±3.5	2.1
		Recovery	167.0±4.5	1.5

* Standard error of the mean.

natant fraction" since the sum of the activities of the two fraction is 81.3 % as compared with the sum for the normal animals of 83.7 %. It is possible that the microsomes, under the stress of a six-day fast, may have undergone some alteration in membrane structure and allowed a seeping of the enzyme into the supernatant. The reason for the normal distribution pattern of the other fractions even after fasting for 6 days is unknown.

The enzyme pattern in set no. 2 is considered to be closer to a true fasting state. All fractions except the microsomes are seen to exhibit increased per cent activities. The recoveries are high (one and one-half times that of the normal).

(iii) Fractionation of Intestines of Rats Fasted 5 Days and Fed Oleic Acid.

Male adult rats were fasted 5 days, fed water ad libitum during this time, and were offered 0.00035 gm-mole oleic acid in 5 grams of wheat gluten. At the end of 6 hours the animals were killed and the intestines fractionated. The results of a typical distribution are shown in Table XIX. It was noted that the enzyme activity of the total homogenate was of the order normally obtained after feeding oleic acid to fasted rats. A high recovery was obtained in this experiment on the basis of homogenate activity equal to 100%. The mean values for four animals are presented in Table XX and these are compared with the

TABLE XIX

DISTRIBUTION OF ALKALINE PHOSPHATASE ACTIVITY IN FRACTIONS ISOLATED FROM HOMOGENATES OF INTESTINES OF RATS FASTED 5 DAYS AND FED OLEIC ACID: A TYPICAL DISTRIBUTION

Fraction	Alkaline Phosphatase (units/gm)	%
Homogenate	96.20	100
Nuclei	11.70	12.2
Mitochondria	27.50	28.5
Microsomes	35.80	37.2
Supernatant	55.70	57.9
Recovery	131.0	136.0

TABLE XX

DISTRIBUTION OF ALKALINE PHOSPHATASE ACTIVITY IN FRACTIONS ISOLATED FROM HOMOGENATES OF INTESTINES IN 0.25 M SUCROSE OF RATS FASTED 5 DAYS AND FED OLEIC ACID

Fraction	Alkaline Phosphatase as % of Homogenate	Factor (comparison with normal)
Homogenate	100	1.0
Nuclei	18.9±2.3 *	1.1
Mitochondria	17.8±4.5	1.5
Microsomes	43.5±3.9	0.8
Supernatant	51.0±3.7	1.6
Recovery	135.0±6.6	1.2

Values are means of 4 determinations.

* Standard error of the mean.

distribution obtained from normal animals. From this table it is seen that the high recoveries persist for animals fed oleic acid after fasting, but are not as great as the values in Table XVIII. The percent activities of the fractions appear to be approaching a normal distribution pattern.

A comparisons of the distributions of the enzyme in three different nutritional states is shown in Table XXI. Rats fasted 5 days and fed oleic acid show a distribution pattern that appears to be intermediate between the patterns exhibited by fasted animals and normal animals. The conclusion is that feeding of fat to starved rats is effective in restoring the normal enzyme levels in the intestine.

The data in Table XXII are intended to indicate which particular fractions undergo alterations in phosphatase activity in response to certain experimental conditions. The values are calculated on the basis of recovery = 100% activity. It is noted that only the microsomes and the soluble fraction manifest changes in activity in following changes in the nutritional environment of the animals. The amount of activity contributed by the nuclei and mitochondria is constant. The loss of activity by the microsomes to fasting is compensated exactly, within the experimental error, by increased activity of the supernatant.

COMPARISON OF THE DISTRIBUTIONS OF INTESTINAL ALKALINE PHOSPHATASE ACTIVITY IN 3 NUTRITIONAL STATES

Nutritional State	No. of Rats	Fractions	% Alkaline Phosphatase
Normal	5	Homogenate	100
		Nuclei	17.0
		Mitochondria	11.8
		Microsomes	52.3
		Supernatant	31.4
		Recovery	112
Fasted 6 Days	4	Homogenate	100
		Nuclei	27.2
		Mitochondria	20.1
		Microsomes	53.3
		Supernatant	66.3
		Recovery	167
Fasted 5 Days and Fed Oleic Acid	4	Homogenate	100
		Nuclei	18.9
		Mitochondria	17.8
		Microsomes	43.5
		Supernatant	51.0
		Recovery	135

TABLE XXII

PHOSPHATASE ACTIVITIES OF THE FRACTIONS IN 3 NUTRITIONAL STATES COMPUTED ON THE BASIS OF RECOVERY EQUAL TO 100

Fraction	Normal	Oleic Acid Feeding	Starved
Recovery	100	100	100
Nuclei	15.0	14.0	16.3
Mitochondria	10.4	13.2	12.0
Microsomes	46.3	32.3	31.9
Supernatant	27.8	37.9	39.7
Micro. + Sup.	74.1	70.2	71.6

From these experiments it appears likely that predominant phosphomonoesterase activity is associated with the microsomes and that it probably constitutes the adaptive portion of rat intestinal alkaline phosphatase which varies with the dietary state as described by Tuba and Robinson (22).

Regarding the question of an activation mechanism (cf. page 66), Schneider (98) has this to say: "In enzyme assays, the possibility that inhibitors or activators are present in the homogenate must be considered. In such a case, the distribution of enzyme and of inhibitor may be entirely different with the result that the sum of the activities of the fractions will be greater or less than the activity of the homogenate. In such cases it is necessary to measure the activities of the fractions in all permutations and combinations as well as separately in order to determine the localization of the inhibitor or activator as well as to eliminate the possibility of denaturation during the isolation procedure". Further work along the lines mentioned above may be warranted in regard to phosphatase of the gut.

3. Discussion

Intestinal alkaline phosphatase of the normal male rat has been found to be associated to a large extent with the"microsome fraction". The conclusion is based upon three findings: a large percentage of the activity of the unfractionated homogenate is found in this fraction; the specific activity of the"microsome fraction" is several times that of the total homogenate; satisfactory recoveries in activities of four fractions were obtained. Relatively little is known about the enzymic properties of microsomes. Omachi (99) worked with mouse liver and found an esterase (methyl butyrase) concentrated in this fraction above that of whole tissue. Hers, Berthet, Berthet, and de Duve (100) presented evidence which suggests that glucose-6-phosphatase and alkaline phosphatase are an integral part of the "microsome fraction" in kidney.

4. Summary

Intestinal cells of the mature male albino rat were completely fractionated by the method of differential centrifugation. The distribution of alkaline phosphatase activity in the cells of the intestine was determined for normal rats, rats starved 6 days, and for rats starved 5 days and fed oleic acid. In the normal animal a large percentage of the total activity of the cell was found to be associated with the "microsome fraction". The specific activity of this fraction was several times that of the total homogenate. In fasting animals the microsomes displayed a decreased activity while the soluble enzyme was found to increase in activity. The activities of these two fractions in intestines of rats fasted 5 days and fed oleic acid tended to return to the pattern displayed by intestines of normal rats. The microsome and supernatant fractions are considered to contain the adaptive portion of rat intestinal alkaline phosphatase.

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